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# Exploiting teeth as a model to study basic features of signaling pathways

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## Abstract

Teeth constitute a classical model for the study of signaling pathways and their roles in mediating interactions between cells and tissues in organ development, homeostasis and regeneration. Rodent teeth are mostly used as experimental models. Rodent molars have proved fundamental in the study of epithelial-mesenchymal interactions and embryonic organ morphogenesis, as well as to faithfully model human diseases affecting dental tissues. The continuously growing rodent incisor is an excellent tool for the investigation of the mechanisms regulating stem cells dynamics in homeostasis and regeneration. In this review, we discuss the use of teeth as a model to investigate signaling pathways, providing an overview of the many unique experimental approaches offered by this organ. We discuss how complex networks of signaling pathways modulate the various aspects of tooth biology, and the models used to obtain this knowledge. Finally, we introduce new experimental approaches that allow the study of more complex interactions, such as the crosstalk between dental tissues, innervation and vascularization.


## Introduction

Teeth are excellent models for studying complexity in biology. From the first stages of tooth development, crosstalk between epithelial and mesenchymal cells initiate the morphological changes that drive tooth formation (1). In adult teeth, homeostasis and regeneration are guaranteed by the cooperation between hard and soft tissues, as well as resident stem cells and their crosstalk with innervation and vascularization (2, 3). The basic features of teeth are highly conserved throughout evolution (4), as tooth-like structures have been discovered in creatures as old as 400million year old, where single tooth plates were used to crush and

shred food (5). In particular, the mechanisms underlying tooth development, homeostasis and regeneration are highly conserved between humans and rodents (6). Rodents possess two classes of teeth: molars and incisors. Rodent molars are homologous to human teeth, and this similarity is reflected in the high conservation of the molecular networks that regulate their development. Rodent and human molars develop through the same morphological stages, and their final anatomy is highly similar, with few morphological and cellular differences (6). The crosstalk between epithelium and mesenchyme that is characteristic of molar development is also the basis for the development of many organs, such as hair follicles, limb, glands, lung and kidney (7, 8). These properties have therefore made the rodent molar an optimal model for the study of morphogenesis and epithelial-mesenchymal interactions. In contrast to molars, rodent incisors grow continuously thanks to the presence of populations of adult dental epithelial (DESCs) and mesenchymal (DMSCs) stem cells (2, 9). As such, the rodent incisor became a preeminent model for the study of stem cells, along with other continuously growing organs such as hairs and nails (10).

The tooth organ is composed of a unique combination of hard and soft tissues. The enamel is the hardest tissue in the human body, and it covers the crown of the tooth (Figure 1). It consists of highly organized hydroxyapatite crystals, as well as by minor fractions of protein matrix and water (11, 12), which are all fundamental for the exceptional mechanical and buffering properties of enamel (13-15). Enamel is supported by dentin, another highly mineralized tissue formed by carbonated hydroxyapatite, collagen, non-collagenous proteins and proteoglycans (Figure 1) (16). The core of the tooth is occupied by the dental pulp, a connective tissue that conveys innervation and vascularization (Figure 1). Nerve fibers from the trigeminal ganglion enter the pulp cavity from the apical foramen and extend until the

dentinal tubules, transmitting pain and sensitivity (17). The dental pulp is highly vascularized, providing oxygen, nutrients and blood-borne factors to sustain tooth survival (18). The tooth is anchored to the surrounding alveolar bone via the periodontium, a unique tissue that offers stability to the tooth by absorbing the masticatory loads (19, 20). Both the dental pulp and the periodontium host resident populations of mesenchymal stem cells that are activated in response to injury (21). All these major anatomical features are shared by human and mouse molars. The tooth integrates the functionality of common tissues present in various organs (e.g. innervation, vascularization), and highly specialized tissues unique to the tooth organ (e.g. enamel, dentin, periodontium). This aspect makes the tooth an exceptional model to study communications between different cell and tissue types, both in physiology and in pathological conditions in which these interactions are disrupted. Moreover, dental pathologies affect the entire population, making the study of tooth biology an important clinical need. Caries leads to the degradation of enamel and, if not properly treated, progress into the dentin and the dental pulp (22). Similarly, the periodontium is often subject to periodontitis, i.e. the infection and inflammation of the periodontium, which is a leading cause of tooth loss (23). Finally, many mutations and syndromes cause dental defects, ranging from tooth agenesis (i.e. lack of teeth) to defective formation of hard tissues (6), all conditions for which satisfactory treatments are not yet available (24).

## Development of the tooth

Tooth development proceeds through a series of well-defined morphological stages both in humans and rodents. Odontogenesis starts during early embryonic development (embryonic day of development 10.5 - E10.5 in mice, 6 weeks in humans) with the primary dental lamina forming as a thickening of oral epithelium at the site of the future tooth row. Dental placodes

form then along the dental lamina (Figure 2) (1). These epithelial structures establish reciprocal interactions with ectodermal-derived neural crest mesenchymal cells, which migrate from the dorsal part of the neural tube (1, 25, 26). Both epithelial and mesenchymal cells possess the capacity to initiate tooth development (27, 28). Recent evidence suggests that a transient epithelial signaling center, called initiation knot, might direct the first phases of tooth development (29). At later stages, the dental mesenchyme maintains the inductive potential, guiding the progression through the sequential stages of odontogenesis (1, 25). The dental placode invaginates into the underlying mesenchyme and forms an epithelial bud (bud stage, E12.5-E13.5 in mice, 6-8 weeks in humans. Figure 2). At this stage, a cluster of non-dividing epithelial cells at the tip of the bud forms the primary enamel knot, a putative signaling center that guides further tooth morphogenesis (8). The continuous proliferation and growth of the dental epithelium around the condensed mesenchyme leads to the epithelial cap configuration (cap stage, E14.5-E15.5 in mice, 11 weeks in humans. Figure 2). The tooth then acquires the bell configuration (E16.5-E18.5 in mice, 12 weeks in humans) as additional signaling centers, named *secondary enamel knots*, start to form in the epithelium in correspondence to the future cusps (8). Between the cap and the bell stages, cytodifferentiation occurs. Four different epithelial layers can be distinguished: outer enamel epithelium (OEE), stellate reticulum (SR), stratum intermedium (SI) and inner enamel epithelium (IEE) (1, 30). At the tip of the cusps, mesenchymal cells from the dental papilla differentiate into odontoblasts that produce and secrete dentin. Shortly after, IEE cells facing odontoblasts differentiate into enamel-secreting ameloblasts (Figure 2). Ameloblasts and all other epithelial cell populations participate in enamel maturation. Upon completion of enamel formation, these cells form the reduced enamel epithelium, which is lost when the tooth erupts into the oral cavity. The dental epithelium is also responsible of the formation

of the roots. The region where IEE and OEE join forms the cervical loop, the site at which root formation is initiated. The IEE and OEE form the *Hertwig's epithelial root sheath* (HERS), which in turn guide root formation (30). The HERS continues to grow apically to shape the root and induces the differentiation of the adjacent dental pulp mesenchymal cells into odontoblasts. These odontoblasts produce the root dentin, while HERS disaggregates into epithelial islands allowing the contact between cells of the dental follicle and the root dentin. HERS cells and mesenchymal cells from the dental follicle cooperate to generate cementum, the hard tissue that allows the anchoring of the tooth roots to the periodontal ligament (31, 32).

In contrast to molars, soon after the bud stage the developing rodent incisors rotate antero–posteriorly and become parallel to the long axis of the jaws. This rotation is accompanied by a morphological reorganization along the labial–lingual axis, as only the labial epithelium will give rise to the enamel-forming epithelium, while the lingual side will form the root analogue (33). Populations of epithelial and mesenchymal stem cells are maintained at the posterior end of the organ and drive its continuous growth (Figure 1). The continuous growth of the rodent incisor is also supported by the intense and continuous remodeling of the periodontium (34). Mature rodent incisors are thus characterized by anatomically distinct and molecularly defined territories, where cell proliferation, differentiation, and maturation events can be easily analyzed (35).

## Signaling pathways and tooth development

All major signaling pathways, i.e. Sonic Hedgehog (SHH), Fibroblast Growth Factor (FGF), Transforming Growth Factor β / Bone Morphogenetic Protein (TGFβ/BMP), WNT, Notch, parathyroid hormone-related protein (PTHrP)/ PTHrP receptor (PPR) (PTHrP/PPR) signaling,

and Hippo-YAP/TAZ pathway are recurrently involved in the regulation of tooth development, from the definition of the dental placodes to the latest stages of mineralization and tooth eruption. Ligands and receptors from the various pathways mediate the interactions between the dental epithelium and the neural crest derived mesenchyme and create molecular gradients as well as barriers within dental tissues that result in the activation of specific genetic programs and transcription factors. The latter are the final mediators of tooth morphogenesis, cytodifferentiation and mineralization.

SHH, FGF, BMP and Wnt signaling cascades are all initiated by soluble ligands, which can signal both locally via autocrine or paracrine mechanisms, or at long distance, creating morphogen gradients (36). SHH signaling is initiated by three possible soluble ligands, Sonic Hedgehog (Shh), Desert Hedgehog (Dhh) and Indian Hedgehog (Ihh). These ligands bind to their receptor Patched1 (Ptch1), relieving Smoothened (Smo) inhibition and ultimately modulating gene expression via Gli transcription factors (37).

The FGF signaling pathway is activated by eighteen secreted proteins that can interact with four tyrosine kinase FGF receptors (FGFRs), which in turn modulate several intracellular cascades, including RAS-MAPK, PI3K-AKT, PLCγ, and STAT pathways. Interaction of FGF ligands with their receptors is strongly regulated by interactions with other proteins and components of the extracellular matrix (38).

TGFβ/BMP signaling is activated by ligands belonging to the TGFβ superfamily, which include TGFβI-III, ten BMPs, and activins, among others (39). TGFβ ligands bind to a large set of receptors (ALK1-7, BMPRI-II, ACTRII/B, TGFβRII), activating the SMAD signaling cascade (39).

153 Wnt signaling is a highly conserved pathway, activated in mammals by 19 different ligands.
154 These ligands interact with Frizzled receptors (FZD), leading to the activation of canonical or
155 non-canonical downstream signaling cascades. Activation of the canonical Wnt pathway
156 results in the nuclear localization of β-catenin, which in turn regulates the transcription of
157 Wnt target genes (40). Non-canonical Wnt signaling results in the activation of JNK, RHOA or
158 PLC downstream effectors (41).

159 The Notch pathway has a different mode of action as its ligands are membrane-bound. This
160 signaling pathway is driven by five trans-membrane-bound ligands (i.e., Jagged1, Jagged2,
161 Delta1, Delta-like3 and Delta-like4) that interact with four Notch receptors (i.e., Notch1,
162 Notch2, Notch3 and Notch4) (42-45). Ligand-receptor interactions trigger the cleavage of the
163 receptor and the subsequent nuclear translocation of its intracellular domain (46). This leads
164 to the activation of Notch downstream genes (44) (47, 48). The Notch pathway thus
165 constitutes a fundamental cell communication mechanism that enables neighboring cells to
166 adopt different fates by creating sharp molecular boundaries (49, 50).

167 The Hippo-YAP pathway perceives and responds to the physical organization of cells in tissues
168 and coordinates these physical cues with classic growth-factor-mediated signaling pathways.
169 The Hippo pathway consists of a core kinase cascade in which the transcriptional co-activators
170 YAP/TAZ are phosphorylated and inactivated by either their exclusion from the nucleus or
171 their enhanced degradation. The nuclear activity of YAP/TAZ regulates cell and organ growth
172 (51).

173 The parathyroid hormone-related protein (PTHrP) is locally acting autocrine/paracrine ligand,
174 which interacts with its receptor, the PTH/PTHrP receptor (PPR), to activate multiple

heterotrimeric G proteins and trigger intracellular signaling pathways . These include primarily Gs/cAMP/protein kinase A (PKA), Gq/PLC/Ca2+/PKC signaling, as well as G12/13/RhoA/phospholipase D and the mitogen-activated protein kinase (MAPK) (extracellular signal-regulated kinase, ERK1/2) signaling cascades (52).

*Initiation*

A precise equilibrium of signals from different signaling pathways establishes the territories where teeth develop, as well as the number of teeth and their class, from very early developmental stages (6, 53). The dental epithelium is already defined before initiation by the expression of the transcription factor *Pitx2* (Figure 2) (54, 55), and its deletion leads to the arrest of tooth development before placode formation (56, 57). The pattern of dentition is also established before the onset of odontogenesis, as competing gradients of FGF and BMP ligands determine the exact localization and class of the different teeth (53, 58-60). BMP4 directs the shape of incisors and FGF8 the shape of molars (53). These ligands induce the expression of specific set of genes in the mesenchyme, namely *Msx1* and *Msx2* in the incisors mesenchyme, and *Dlx1* , *Dlx2* and *Barx1* in the molars mesenchyme (Figure 2) (1*)*. The specific combination of these transcription factors determines the development of the tooth germs toward an incisorform or molariform shape (61). SHH and FGF signaling are fundamental drivers of the early phases of odontogenesis. *Shh* is expressed in the dental epithelium at E11.5, and *Shh*-expressing cells attract *Fgf8*-expressing epithelial cells to the dental placode (62). Both these pathways are necessary for the progression of odontogenesis, as their inhibition leads to the arrest of tooth development (62, 63). Wnt/β-catenin also plays a major role in the initial phases of tooth development. Inhibition of epithelial Wnt signaling blocks tooth development at the placode stage (64), while inhibition of mesenchymal Wnt signaling

arrests odontogenesis at the bud stage (65). Conversely, constitutive activation of Wnt/β-catenin in the oral epithelium induces the formation of ectopic teeth (66, 67).

*Morphogenesis*

At the bud stage, FGF and BMP signaling are needed to induce the expression of the transcription factors *Pax9* and *Msx1* in the dental mesenchyme. Pax9 and Msx1 themselves regulate the expression of *Fgf3*, *Fgf10* and *Bmp4* (68, 69), mediating the progression of odontogenesis (Figure 2). At the bud-cap transition, the dental epithelium is characterized by the appearance of the enamel knots, putative signaling centers localized in correspondence of the future cusps (8). Cells within the enamel knot do not proliferate and produce WNTs, SHH, BMPs and FGFs, which in turn modulate the proliferation of adjacent epithelial cells, leading to the formation of dental cusps (Figure 2) (70). Notch signaling also plays an important role in the determination of the number and shape of cusps. The Notch ligand Jagged2 (Jag2) is expressed in the dental epithelium under the control of mesenchyme-derived FGFs and BMPs, and loss of Jag2 leads to the formation of smaller cusps via deregulation of *Bmp4*, *Pax9* and *Barx1* expression (71). Enamel knots and cusp formation are also strongly modulated by the activity of the TNF family ligand ectodysplasin A (EDA). The expression of *Eda* is regulated by Wnt signaling, and EDA in turn regulates the expression of *Fgf20 (72)*. Mice carrying *Eda* deletions have smaller enamel knots, defective dental epithelial invagination and flat cusps (73). During the cap and bell stages, proliferation of the progenitors residing in the cervical loops sustain the elongation of the dental epithelium. FGF10 from the dental mesenchyme plays an important role in this process, as loss of FGF10 impairs the generation of new epithelial progenitors and induces apoptosis of cells located in the cervical loops (Figure 2) (74).

*Cytodifferentiation and mineralization*

A key event that characterizes the cap and bell stages is cytodifferentiation. Dental epithelial cells differentiate into ameloblasts, which form enamel, the hardest mineralized tissue of the body. The specification of these cells involves a complex crosstalk between FGFs, BMPs, SHH, the Notch signaling pathway (71, 75, 76) and the transcription factor Tbx1 (Figure 2) (77). FGF is required for the generation of ameloblasts progenitors (74), while SHH activity is necessary for the ameloblasts differentiation (78). Notch signaling plays a key role at this stage. During epithelial cytodifferentiation, Notch receptors and their ligands are expressed in adjacent cells of the dental epithelium, creating sharp boundaries that define dental epithelial cell identity (71, 75, 76, 79, 80). The expression of Notch ligands and receptors in the dental epithelium is modulated by FGF and BMP ligands (71, 80, 81), and Notch signaling modulates the differentiation of the various dental epithelial lineages (71, 82). The Notch ligand Jag2 is expressed in the dental epithelium, and its deletion impairs ameloblastic differentiation and enamel production (71). This effect is partially mediated by the loss of the expression of Tbx1 (77). Tbx1 mutations in human patients are associated with DiGeorge Syndrome, a complex human pathology characterized by craniofacial malformations and defective enamel formation. Mice lacking Tbx1 do not have enamel, as ameloblasts fail to differentiate (77).

Signaling pathways are also directly involved in amelogenesis, i.e. the formation of enamel. Shh activity is required for the proper terminal differentiation of ameloblasts (78). Wnt/β-catenin signaling is essential for ameloblast movement during differentiation (83), while constitutive activation of β-catenin in ameloblasts delays their differentiation and leads to the generation of hypomineralized enamel (84). Bcl9 and Pygo2, which are generally considered to be Wnt/β–catenin transcriptional cofactors, are necessary for the fine

structure of enamel, as their deletion leads to the formation of defective enamel. In ameloblasts, these two molecules directly interact with amelogenin and other important enamel proteins, modulating their localization and thus enamel structure (Figure 2) (35).

*Root formation*

Root development strictly relies on the interaction between epithelial cells from HERS and mesenchymal cells from the dental papilla and the dental follicle (32). Several signaling pathways are involved in this process. Bmp ligands are expressed in the early phases of root formation, and their crosstalk with Shh signaling is necessary for proper HERS formation and root development. Loss of Bmp signaling in the dental epithelium leads to the persistence of Shh-dependent signaling activity in the root, leading to the inhibition of HERS formation and shorter roots (32, 85). The disappearance of Fgf10 expression in the dental mesenchyme is necessary for the initiation of root formation, while exogenous Fgf10 application at the crown-to-root transition inhibits HERS formation (32, 86). Wnt signaling is also fundamental in this process. *Wnt10a*-null mice are affected by taurodontism, a condition characterized by larger pulp chambers and shorter and poorly furcated roots, similar to patients with *WNT10A* mutations (87, 88). Similar phenotypes are induced by the constitutive activation of Wnt signaling in dental follicle cells (89). Recent works highlighted the importance of the PTHrP/PPR signaling axis (52) in the formation of the roots, as deletion of the PPR receptor leads to failure of eruption and the development of significantly truncated roots, which lack periodontal ligaments (90, 91), similar to what observed in human patients with *PTHR1* mutations (92).

*Homeostasis and regeneration*

Dental stem cell behavior in homeostasis and regeneration is mostly studied in rodent incisors. A complex network of signaling pathways, including FGF, BMP, Notch, and SHH, regulates the maintenance and the differentiation of DESCs and DMSCs. FGF10 and FGF3 are produced in the mesenchyme and promote both the survival and differentiation of DESCs (74, 93). FGF9 is produced within the dental epithelium and directly regulates Shh expression, which together with other Hh ligands modulates both DESCs proliferation and differentiation (78, 94). In contrast, BMP and Wnt signaling have antiproliferative and proapoptotic effects on DESCs (95). Incisor DMSCs activation and subsequent differentiation is modulated by nerve-derived Shh (2) as well as Notch signaling (96). Both epithelial and mesenchymal stem cells are essential not only in homeostasis but also during injury repair. Injury to the incisor epithelium leads to the activation of stem and progenitor cells characterized by the expression of Notch1-expressing cells, which contribute to the regeneration of the damaged epithelial layers (82, 97). Recently, Hippo signaling has also been shown to play an important role in regulating DESCs proliferation and differentiation. Hippo-effectors Yap and Taz prevent premature differentiation of DESCs and transit amplifying progenitors via activation of the mTOR signaling pathway, and their deletion leads to loss of cell proliferation and increased cell death in the labial cervical loop area (98).

## Teeth as experimental models

The tooth constitutes a model of excellence to study all the basic processes that underlie organ development, from initiation to morphogenesis, terminal differentiation and mineralization, as well as homeostasis and regeneration (Figure 3). The non-lethality of dental defects and diseases in animal models make teeth an exceptional model for the study of the roles of the different signaling pathways *in vivo*. Defects caused by gene deletions are easily

289 detected in teeth, in particular those that result in arrest of tooth development or in
290 alterations of enamel formation (35).

291 *Developing teeth as prototypical model for epithelial-mesenchymal interactions*

292 The first phases of molar development are strictly controlled by epithelial-mesenchymal
293 interactions. Classical recombination experiments were fundamental to determine that the
294 odontogenic potential, i.e. the ability to induce formation of teeth, shifts between the
295 epithelium and the mesenchyme through development (25, 28, 99). Heterochronic
296 recombination experiments showed that the inductive potential resides in the dental
297 epithelium between E9 and E11, and then it shifts to the dental mesenchyme (25, 28).
298 Heterotypic recombination of dental mesenchyme, isolated from molars beyond E11.5, with
299 non-dental epithelium (e.g. isolated from hair follicles) invariably resulted in the generation
300 of teeth, showing that the dental mesenchyme contained all the signals required to induce
301 odontogenesis (25). Moreover, *In vivo* recombination experiments showed that prior to
302 initiation, the neural-crest derived mesenchyme has the potential to induce the odontogenic
303 program into the oral epithelium (27). Indeed, tooth-like structures developed in mouse-
304 chicken chimeras, where neural crest cells from chicken embryos were replaced by mouse
305 crest cells. This indicates that cranial neural crest cells contain inductive potential and,
306 together with the oral epithelium, contribute to the initiation of tooth formation (Figure 3)
307 (27, 100).

308 *Tooth germ cultures*

309 Embryonic mouse tooth germs can be isolated and cultured as organs in high serum-
310 containing media, as signals exchanged between the epithelium and the mesenchyme allow

311 the progression of tooth development *in vitro* (101). The development of cultured tooth
312 germs is comparable to the *in vivo* tooth development both in terms of structure and function
313 (Figure 3) (101). Tooth organ cultures have thus proved fundamental to study the roles of
314 signaling pathways in the modulation of tissue morphogenesis and cytodifferentiation, as
315 these organotypic cultures allow live imaging (97) as well as easy treatment of the developing
316 tooth germ by electroporation (102), as well as with antibodies, beads releasing growth
317 factors (Figure 3), and small molecules (71, 77, 103-105). Tooth organ cultures lack however
318 important elements such as innervation and vascularization, which are key modulators of
319 many processes, including mineralization and stem cells activation (2, 3). After *in vitro*
320 treatments, cultured tooth germs can be transplanted subcutaneously or in the kidney
321 capsule. In these conditions, tooth germs can receive blood-derived nutrients and signals that
322 are necessary for the full formation of the mineralized tissues (101).

323 *Teeth as model for the study of genetics, genetic therapy and regeneration*

324 Rodent molars constitute optimal models to study the molecular and genetic bases of human
325 tooth development and disease. Mutations affecting tooth development in human patients
326 lead to homologue phenotypes in mice, and most of our knowledge concerning tooth
327 development was obtained via studies on mouse models (6, 88, 106). The existence of
328 discrete morphological stages in odontogenesis provides an easy read out for developmental
329 phenotypes, such as tooth agenesis or arrest of tooth development, induced by alterations of
330 specific signaling pathways (35, 102, 107, 108). In addition, diseases characterized by
331 defective enamel formation, e.g. Amelogenesis Imperfecta, are properly modeled in animal
332 models (6, 109-111). The rodent molar periodontium has composition and behavior similar
333 to the periodontium of human teeth, and has thus been used as a model to study periodontal

pathologies and the responses of periodontal structures to mechanical stress (34). Based on this knowledge, tooth organ cultures can then constitute an optimal platform to investigate the genetic treatment of these pathologies via e.g. electroporation (112).

Both human and rodent teeth host populations of epithelial and mesenchymal stem cells, which have attracted great attention for their clinical potential. Molars host DMSCs both in the dental pulp and in the periodontium, located in close association with blood vessels (21, 113-115). These cells are multipotent, have strong angiogenic and neuroattractive properties (116-118), and are activated in response to injury and other external stimuli (21, 114, 119). Many studies focused on their use in combination with scaffolds to promote regeneration of the dental pulp and the periodontium (117, 120-122). Rare DESCs have been isolated from the periodontium of human teeth and are regarded with great interest for the regeneration of enamel (123). Properties of dental stem cells are however mostly studied in rodent incisors. Incisors grow continuously thanks to the crosstalk between epithelial and mesenchymal stem cell niches, as well as due to their interaction with nerve fibers and blood vessels (2, 9). Lineage tracing and conditional knockout approaches allow the study of the effects of signaling pathways on stem cell function *in vivo* (90, 91, 97, 124). Moreover, the rodent incisor is easily accessible and the cutting of its anterior end does not evoke pain in the animals, thus allowing lowly invasive studies of stem cells reaction to injury (96, 97). Exogenous stem cells can also be injected close to the posterior end of the incisor (125). The periodontium of the rodent incisors remodels continuously to support the continuous growth of the organ, showing a more dynamic behavior than that observed in human teeth and mouse molars (34). Similar to molars, incisors can be cultured in high serum-containing media, as their stem cell populations continue to proliferate and differentiate *in vitro* (126-128). This is turn allows

efficient live imaging, treatments and injury studies (97, 102). The rodent incisor is thus an exceptional model to study the roles of the various signaling pathways on DESCs and DMSCs, both in homeostasis and regeneration.

Three-dimensional culture systems started to be used to generate dental tissues starting from single cells. Dental spheroids (dentospheres) were successfully obtained from mouse and human dental epithelial and mesenchymal stem cells (Figure 3) (129-132). Depending on the culture conditions, epithelial dentospheres generated from mouse incisors and molars either showed strong stem cell properties or generated structures characterized by differentiation gradients (129). These dentospheres were shown to model, to a certain extent, some of the basic molecular networks underlying ameloblastic differentiation (132). Dental epithelial and mesenchymal cells can also be combined in three-dimensional systems to generate bioengineered tooth germs (133). Bioengineered teeth have been generated from mouse embryonic dental mesenchymal and epithelial cells. These teeth contained the various dental tissues, while their size was significantly inferior to that of normal teeth (133). To date, no biologically functional bioengineered tooth has been generated from adult cells.

*"Organ-on-a-chip": modeling the interactions between the different dental tissues*

Monocultures, organoids and spheroids lack many features necessary for the function of any organ, such as vasculature, innervation, mechanical cues, and immune responses (134). "Organ-on-chip" systems aim to fulfil this need by co-culturing epithelial, mesenchymal, endothelial, and neuronal cells or tissues in communicating chambers (135). Microfluidic devices (Figure 3( have already been used to co-culture developing tooth germs (136), dental stem cells (118), and dental cancer stem cells (137) with trigeminal ganglia, the main mediator

of tooth innervation. These studies showed that microfluidics can faithfully recapitulate the *in vivo* situation, thus providing a solid ground for the study of dental tissues in "organ-on-chip" systems (119).

## Perspectives

- **Highlight the importance of the field**. In this review we discuss the use of teeth as models to study signaling pathways in organ development, homeostasis and regeneration. Teeth are indeed a prototypical model for the study of morphogenesis, epithelial-mesenchymal interactions, mineralization, and stem cell biology.
- **A summary of the current thinking.** Combination of powerful *in vivo* (e.g. transgenic animals) and *in vitro* (e.g. tissue recombinants, long-term whole organ culture) approaches make teeth a unique model for the study of signaling pathways. Complex networks of signaling cascades are involved in tooth initiation, morphogenesis, cytodifferentiation, mineralization, as well as injury and regeneration. Study of these processes has provided important information concerning basic features of signaling pathways, as well as fundamental input for their exploitation for clinical purposes.
- **A comment on future directions.** The recent technological advances in three-dimensional culture systems, microfluidic "organ-on-a-chip" platforms, gene editing techniques and nanotechnology are providing new possibilities for the study of signaling cascades and of interactions between the different dental tissues. Moreover, they offer exciting perspectives for regenerative and personalized medicine.

## Author contributions

PP, CP, and TM contributed to the writing, reading, and editing of the present review article. All authors have read and agreed to the published version of the manuscript.

## Declaration of interests

The authors declare no competing interests.


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## Figures and Figure legends

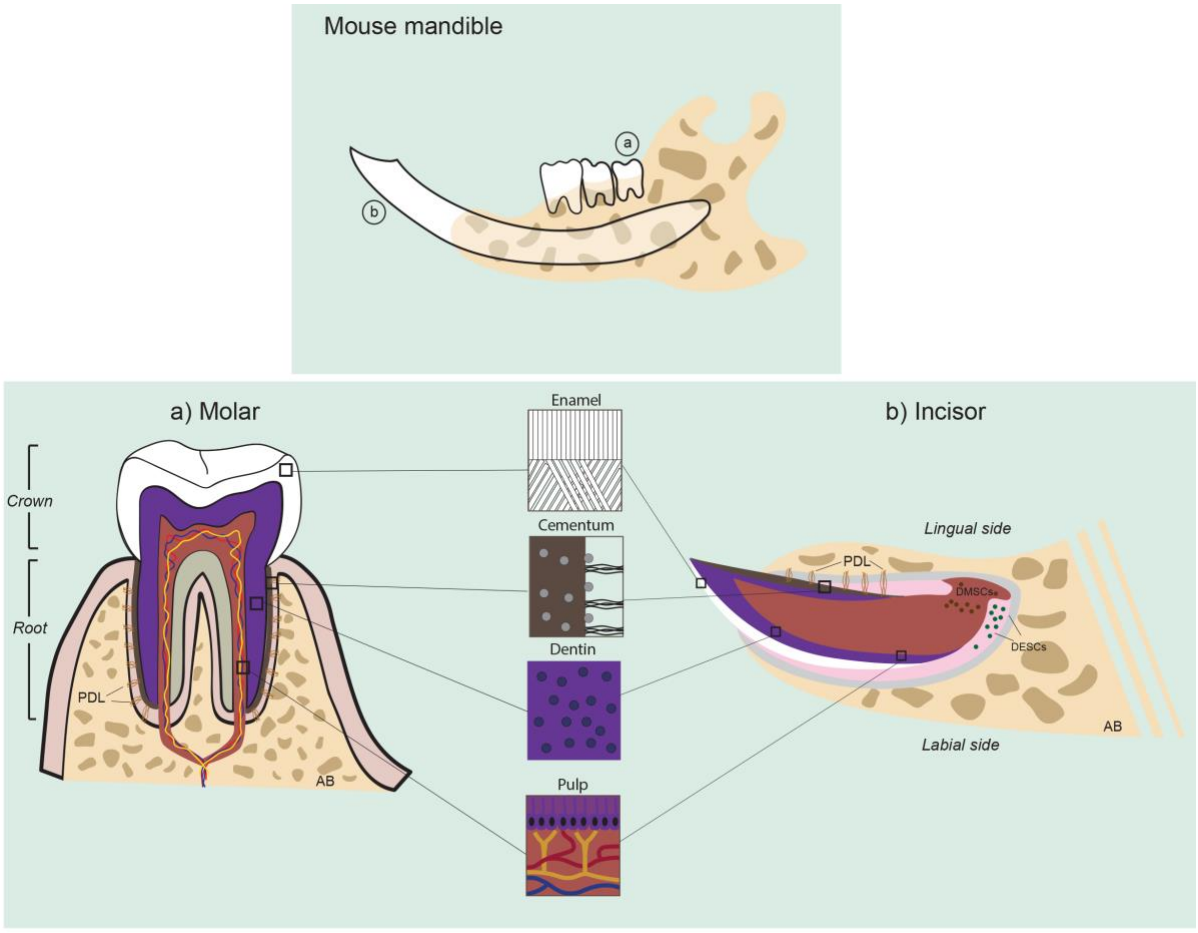
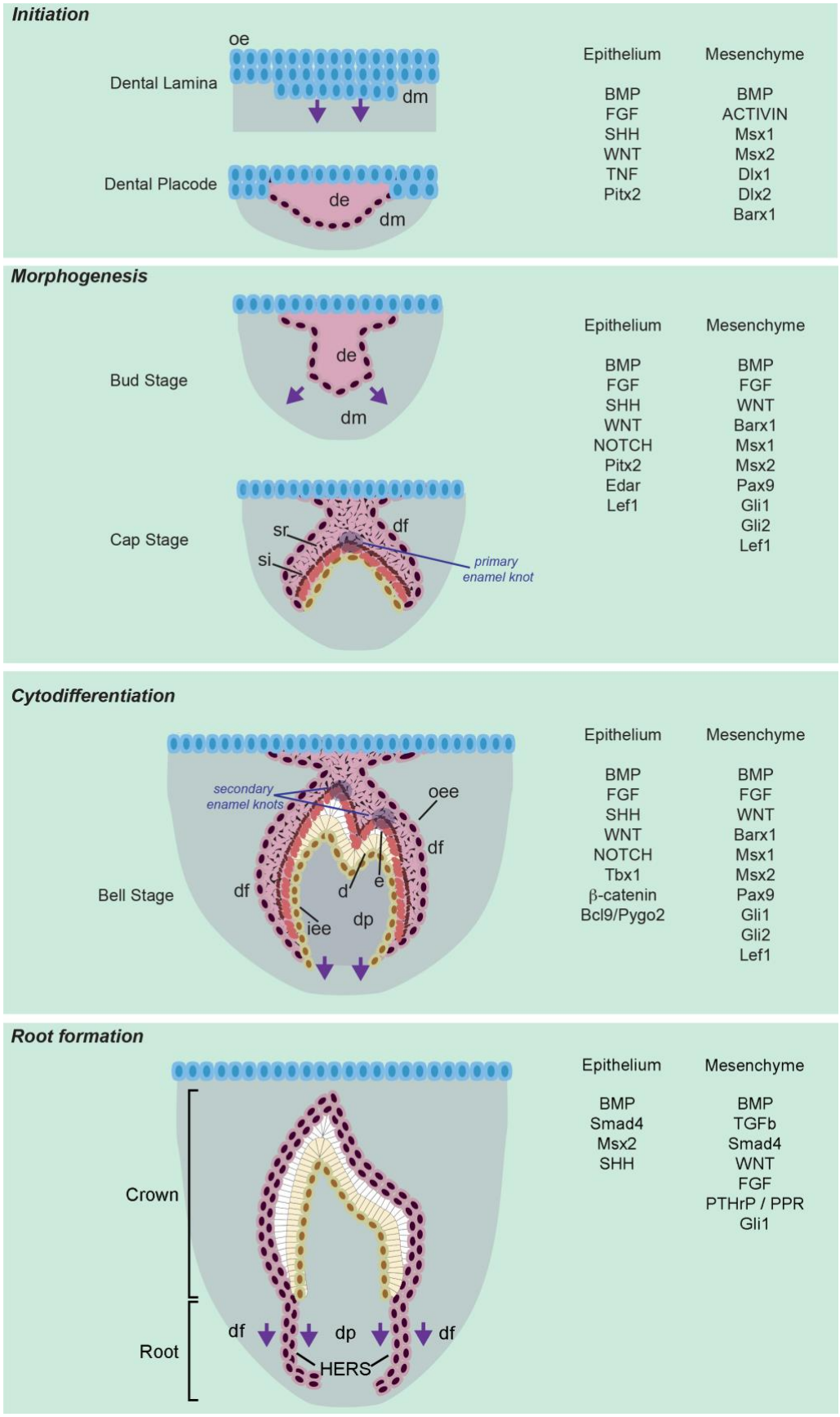



**Figure 1.** Schematic representation of the anatomy of rodent molars and incisors. Abbreviations: AB, alveolar bone; DESCs, Dental Epithelial Stem Cells; DMSCs, Dental Mesenchymal Stem Cells; PDL, periodontal ligament. Above: general structure of a mouse lower hemi jaw, and relative localization of the molars and the continuously growing incisor. Below, a): Structure of human molar. The overall features are conserved between human and rodent molars. b) Structure of the continuously growing rodent incisor.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Mesenchyme
BMP
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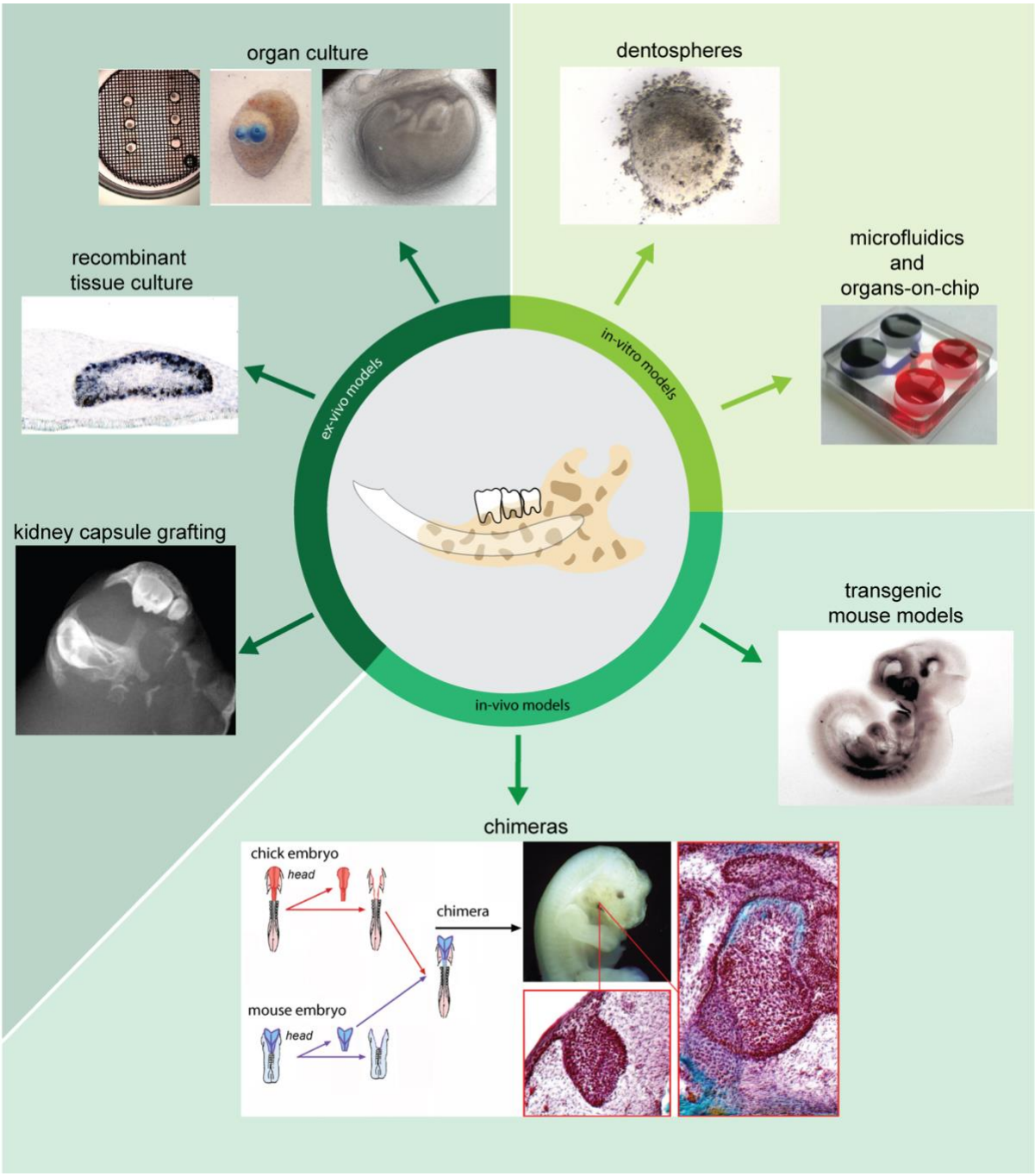
WNT
Barx1
Msx1
Msx2
Pax9
Gli1
Gli2
Lef1
Cytodifferentiation
Bell Stage
secondary enamel knots
oee
df
df
d
e
dp
iee
Epithelium
BMP
FGF
SHH
WNT
NOTCH
Tbx1
β-catenin
Bcl9/Pygo2
Mesenchyme
BMP
FGF
WNT
Barx1
Msx1
Msx2
Pax9
Gli1
Gli2
Lef1
Root formation
Crown
Root
df
dp
df
HERS
Epithelium
BMP
Smad4
Msx2
SHH
Mesenchyme
BMP
TGFb
Smad4
WNT
FGF
PTHrP / PPR
Gli1

418

**Figure 2**. Overview of tooth development. The main signaling pathways (capital letters) and transcription factors (lowercase) involved at the different morphological stages are listed. Abbreviations: d, dentin; de, dental epithelium; df, dental follicle; dm, dental mesenchyme; dp, dental papilla; e, enamel; HERS, Hertwig's Epithelial Root Sheath; iee, inner enamel epithelium; oe, oral epithelium; oee, outer enamel epithelium.




425

426 **Figure 3.** Overview of the main *in vivo*, *ex vivo* and *in vitro* experimental approaches for the

427 study of signaling pathways in dental tissues.

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